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Award Number: DAMD17-02-1-0562

TITLE: Blocking Blood Supply to Breast Carcinoma with a DNA Vaccine Encoding VEGF Receptor-2

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REPORT DATE: April 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Annual (15 Mar 04 - 14 Mar 05)

4. TITLE AND SUBTITLE
Blocking Blood Supply to Breast Carcinoma with a DNA Vaccine Encoding VEGF Receptor-2

5. FUNDING NUMBERS
DAMD17-02-1-0562

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Our major tasks in this last grant year focused on gaining insights into CTL-mediated immune mechanisms induced by FLK-1 whole gene and minigene-based DNA vaccines against breast tumors. We also extended our efforts to a new area of breast cancer research by targeting the transcription factor Fos-related antigen 1(Fra-1), overexpressed by breast carcinoma. As mentioned in last year's progress report, we extended the last grant period by one more year because of these additional exciting and novel findings. We report for the first time on the anti-breast carcinoma activity of antiangiogenic DNA minigene vaccines and identify the first H-2 Db-restricted FLK-1 epitope-FLK_{400} (VILTNPISM). Importantly, the pHI-Db and pHI-FLK_{400} minigene vaccines achieved similar efficacy as the DNA vaccine encoding the full length FLK-1 gene. They present a much simpler and more manipulatable alternative to the whole gene vaccine and add a new dimension to anti-angiogenic interventions in breast cancer. In addition, we also demonstrated that immunization with a DNA vaccine encoding murine Fra-1, co-transformed with a gene encoding secretory murine cytokine IL-18, can effectively eradicate two different breast cancers, 4T1 and D2F2 in syngeneic mouse models and generate a long-lived specific tumor-protective immune response.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
58

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

A successful treatment leading to the eventual elimination of breast cancer represents a considerable research challenge since there is currently no cure for locally advanced or metastatic breast cancer. It is for this reason that we proposed to develop a novel genetic therapy by constructing a DNA vaccine based on the VEGF receptor-2 (FLK-1). This receptor tyrosine kinase is overexpressed on proliferating endothelial cells and this provides a useful target for antiangiogenic therapy in the tumor microenvironment. In order to break peripheral T cell tolerance and optimize a T cell-mediated immune response against the FLK-1 self-antigen, a small viral peptide HIVtat, one of the commonly used membrane-translocating peptides, was fused to FLK-1 peptides in order to facilitate effective delivery of our minigene vaccines to secondary lymphoid tissues assuring effective activation of both antigen-presenting dendritic cells (DCs) and naïve T cells.

The rationale for selecting transcription factor Fos-related antigen 1 (Fra-1) as a DNA vaccine target was to extend our research effort as follows: First, Fra-1 is overexpressed by many human and mouse epithelial carcinoma cells, especially those of breast cancer; Second, this transcription factor is involved in the progression of various breast cancer cell types, and thus represents a relevant, potential target for a breast carcinoma vaccine. Finally, it is a novel approach to target a transcription factor by using a DNA vaccine strategy. In addition, IL-18 is a well-known multi-functional cytokine that was co-expressed in our DNA vaccine in order to enhance Fra-1 antigen presentation by DCs to naïve T cells, and thereby induce an antitumor immune response. IL-18 was originally believed to elicit cytokine production by T and/or NK cells and to induce their proliferation and cytolytic activity, similar to an IFN-γ-inducing factor. However, the

antitumor activity attributed to IL-18 is now considered to be primarily mediated by T and NK cell activation and by enhancing cellar immune mechanisms via up-regulation of MHC class I antigen expression, favoring the differentiation of $CD4^+$ helper T cells toward the T helper 1 (Th1) subtype.

Taken together, in this fiscal year, we mainly focused on the following tasks of our grant proposal; 1) To evaluate the *in vivo* antiangiogenic/antitumor activities of the FLK-1-based DNA vaccine and of $H\text{-}2K^d$ and/or $H\text{-}2D^d$ FLK-1 minigene vaccines in orthotopic and experimental 4T1 and D2F2 mouse mammary tumor metastasis models in syngeneic BALB/c mice; 2) To critically delineate CTL-mediated immune mechanisms induced by the FLK-1-based DNA vaccines with emphasis on the working mechanism of minigene vaccines, including the capability of CTLs induced by the DNA vaccine to induce killing of proliferating endothelial cells and activation of T cell functions involved in tumor protective immunity with emphasis on T cell specific cytotoxicity and determination of T cell memory; 3) To test the possibility that a Fra-1 DNA-based vaccine can induce, both antitumor immunity and long-lived T cell memory, after oral vaccination and to gain insights into the immunological mechanisms involved in T cell activation and memory induction.

BODY:

As mentioned above, our major tasks were to determine antitumor effects as well as suppression of angiogenesis induced by either minigene-based DNA vaccines encoding nonapeptide minigenes with either H-2K^d and/or H-2D^d anchor residues, or the entire FLK-1 gene and to gain insight into critical involvement of immunological mechanisms. Thereafter, we proved the following hypotheses and accomplished these tasks:

1) A DNA vaccine targeting Fos-related antigen 1 enhanced by IL-18 induces long-lived T cell memory against tumor recurrence.

As mentioned in our task, we extended our prior studies on a Fra-1 based DNA vaccine co-expressing IL-18 in two breast tumor models, 4T1 and D2F2, by investigating potential working mechanisms of this vaccine, focusing particularly on the generation, function and long-term survival of CD8$^+$ memory T cells in tumor models of syngeneic BALB/c and SCID mice after adoptive transfer of T cells from successfully vaccinated mice. We also focused on CD8$^+$ T cells that could remain dormant at high frequency in non-lymphoid tissue after successful vaccination, because their ultimate presence in the periphery is important for eliciting resistance against secondary tumor cell challenges. These questions were addressed with a polyubiquitinated DNA vaccine encoding Fra-1, co-transformed with secretory murine cytokine, IL-18 and carried by attenuated *Salmonella typhimurium* to secondary lymphoid tissues, which proved capable of inducing a long-lived CD8$^+$ T cell response. (Please view the attached paper for details.)

2) Generation of FLK-1minigene vaccines encoded by expression vectors.

DNA minigene vaccine approaches were adopted in order to identify specific CTL epitope(s) to facilitate in depth mechanism studies and to test our hypothesis that vaccination with such

epitope(s) can induce immune responses similar to those achieved by the whole FLK-1 gene vaccine. To this end, three peptides were included in H-2 Db-restricted or H-2 Kb-restricted minigenes based on their predicted binding to MHC class I molecules.

To achieve optimal vaccine efficacy, expression vectors were constructed based on the backbone of pCMV/Myc/ER (Figure 1A). In order to check gene expression, 293T cells were transfected with either pHI-myc, pHI-Db-myc or pHI-Kb-myc. Protein expression was assessed by Western blotting and single bands with the expected molecular mass of 15KDa were detected (Figure 1B). The vaccine vectors pHI, pHI-Db and pHI-Kb were generated by introducing a stop codon immediately downstream from the peptide coding sequences, so that mature peptides did not contain the myc epitope. Structures were confirmed by DNA sequencing and empty pCMV vectors were also included for control purposes.

3) The pHI-Db vaccine induces a FLK_{400}-specific immune response.

To validate that the FLK_{400}-specific immune response contributes to the antitumor effects elicited by the entire FLK-1 gene, a pHI-Db vaccine, and a minigene vaccine encoding only FLK_{400} in the absence of FLK_{94} and FLK_{1210} epitopes were used to compare their antitumor effects with those of pHI-Db and the entire FLK-1 gene in an EO771 breast carcinoma model in syngeneic C57BL/6 mice. Both pHI-Db and pHI-FLK_{400}-based vaccines significantly protected these mice and to an extent comparable to that induced by DNA vaccine encoding the entire FLK-1 gene (Fig 2 A,B). Accordingly, our data suggest that FLK_{400} is the major epitope which mediates the antiangiogenic effects induced by the pHI-Db minigene vaccine.

4) The FLK-1-based DNA minigene vaccine pH1-D^b induces specific CTL killing.

To assess the specificity of the CTL-mediated killing of tumor cells, splenocytes from vaccinated mice were stimulated with synthetic peptides for 5 days and tested in cytotoxicity assays against

the murine endothelial cell line, MS1 and the prostate cancer cell line, RM9 targets in a cytotoxicity assay. The FLK_{400}-based minigene vaccine stimulated splenocytes that exhibited specific cytotoxic killing against the MS1 endothelial cell target, but showed only background killing of RM9 prostate cancer cells (Fig 3B). FLK_{94}-stimulated splenocytes revealed only low level of MS1-specific killing (Fig 3C), while FLK_{1210}-stimulated splenocytes displayed only low level or non-specific cytotoxic killing (Fig 3D).

The peptide-stimulated splenocytes isolated from mice vaccinated with pHI-Db were twice restimulated *in vitro,* weekly, with irradiated, peptide-loaded splenocytes, and tested again for their cytotoxicity. In fact, splenocytes restimulated with FLK_{400}-loaded splenocytes showed greatly enhanced cytotoxicity, e.g. a higher % specific killing at a much lower E:T ratio (Fig 3E). Restimulation with FLK_{94}-loaded splenocytes resulted in a lower level of MS1-specific killing (Fig 3F), while splenocytes stimulated with FLK_{1210}-loaded peptide failed to show any significant cytotoxic killing (Fig 3G). These data suggest that restimulation with FLK_{400}-loaded splenocytes enriches the T cell population which is specific for endothelial cells and indicates that FLK_{400}-specific CTLs are responsible for the specific killing of proliferating endothelial cells in the tumor microvasculature.

5) A DNA vaccine encoding full length FLK-1 induces FLK_{400}-specific responses. FLK_{400}-specific responses were also detected in mice vaccinated with pFLK-1 demonstrated by ELISPOT assays (Fig 4A, B). Splenocytes from these mice also displayed preferentially killed EO771 breast tumor cells loaded with FLK_{400}, when compared to the killing of unloaded EO771 cells (Fig 4C). This same kind of cytotoxic killing was also observed in pHI-Db-vaccinated mice (Fig 4D), that served as positive controls. The killing of wild-type EO771 cells or of FLK_{400}-loaded EO771 cells was largely indistinguishable in the pCMV or pHI control groups (Fig 4E

and F). Taken together, these data prove that FLK_{400}-specific responses were also induced by the DNA vaccine encoding the entire FLK-1 gene.

6) FLK-1 based vaccination suppresses angiogenesis in the 4T1 breast cancer model. Matrigel assays were employed to verify the antiangiogenic effect of the pHI-Db minigene vaccine. In fact, vaccination with pHI-Db suppressed vascularization, as demonstrated when representative Matrigel plugs placed into vaccinated mice were removed after *in vivo* staining of endothelium with FITC-conjugated lectin (Fig 5A) and by their relative fluorescence (Fig 5B). Taken together, these findings demonstrate that the pHI-Db minigene protects mice from breast tumor cell challenges via antiangiogenic effects.

KEY RESEARCH ACCOMPLISHMENTS:

1) In the Fra-1 project, we demonstrated that a novel vaccination strategy against a transcription factor induced specific $CD8^+$ T cell-mediated immunity that eradicated spontaneous and experimental breast cancer metastases. Insights into the immunological mechanisms involved primarily robust tumor-specific cytotoxicity and production of long-lived T cell memory.

2) We demonstrated marked antitumor effects in our FLK-1 research project including the development of the first antiangiogenic FLK-1 minigene vaccines and identified the first H-$2D^b$-restricted FLK-1 epitope-FLK_{400} (VILTNPISM). Importantly, the pHI-Db and pHI-FLK_{400} minigene vaccines achieved similar efficacy as the DNA vaccine encoding the full length FLK-1, thereby providing a much simpler and more manipulatable alternative to the whole gene vaccine, and adding a new dimension to antiangiogenic interventions against breast cancer.

REPORTABLE OUTCOMES:

The reportable outcomes and results from this fiscal year of this grant are as follows:

1) A manuscript entitled "A DNA vaccine targeting Fos-related antigen 1 enhanced by IL-18 induces long-lived T cell memory against tumor recurrence" has been accepted by CANCER RESEARCH and will be published by the middle of this year.

2) A manuscript named "T cell-mediated suppression of angiogenesis results in tumor protective immunity" has been finished and will be sent to "PNAS" for peer review.

CONCLUSIONS:

We proved the major hypothesis driving this project that effective regression of breast cancer growth and metastases by suppressing tumor angiogenesis and disseminating metastases with an orally delivered DNA vaccines carried by double attenuated *Salmonella typhimurium* encoding either the entire FLK-1 gene, FLK-1 minigenes or the Fra-1 gene. The key accomplishments of this final grant period can be summarized as follows: 1) We demonstrated that an oral vaccine encoding Fra-1 and IL-18 genes carried by an attenuated strain of *Salmonella typhimurium* to secondary lymphoid tissues protected Balb/c mice against a lethal challenge of murine D2F2 and 4T1 breast cancer cells. Furthermore, we delineated for the first time an in-depth mechanism indicating that IL-18 plays a key role in maintaining T cell memory but does not induce it. All findings have been summarized in a manuscript that was accepted by CANCER RESEARCH. In addition, regarding our FLK-1 gene vaccine project, we could report that anti-breast cancer effects were achieved for the first time by an oral DNA minigene vaccine against murine vascular endothelial growth factor receptor-2 (FLK-1), a self-antigen specifically overexpressed by proliferating endothelial cells in the tumor vasculature. Moreover, we identified the first H-$2D^b$-restricted epitope-FLK_{400} (VILTNPISM), specifically recognized by CTLs which killed FLK-1^+ endothelial cells, resulting in suppression of angiogenesis and long-lived protection against breast cancer. The specificity of this immune response was indicated as the DNA vaccine encoding the entire FLK-1 gene also induced a FLK_{400}-specific CTL response. These research findings have led to a manuscript entitled, "T cell-mediated suppression of angiogenesis results in tumor protective immunity", which will be submitted shortly for peer review.

APPENDICES:

Fig. Legend

Fig. 1

Fig. 2,

Fig. 3

Fig. 4

Fig. 5

Publication:
Luo, Y., Zhou, H., Mizutani, M., Mizutani, N., Liu, C., Xiang, R. and Reisfeld, R.A. A DNA vaccine targeting Fos-related antigen 1 enhanced by IL-18 induces long-lived T cell memory against tumor recurrence.

Figure Legends

Figure 1. Construction of FLK-1 minigene DNA vaccine. (A) Schematic map: Minigenes encoding HIVtat translocation peptide, a spacer, and murine FLK-1 H-2Db- and Kb-restricted epitopes were assembled by PCR with overlapping oligonucleotides as templates. Db-restricted epitopes include FLK_{94}: RVVGNDTGA; FLK_{400}: VILTNPISM; FLK1210: FHYDNTAGI. Kb-restricted epitopes include FLK_{54}: RGQRDLDWL; FLK_{771}: VIAMFFWLL; FLK_{1129}: TTPEMYQTM. The PCR fragments generated were cloned into a pCMV vector by using *Bss*H II and *Xho* I restriction sites. (B) Proteins encoded by minigenes were expressed in mammalian cells. This was indicated when 293T cells were transfected with either pHI-Db-myc or pHI-Kb-myc for 24 hours, harvested, lysed and analyzed by Western blotting with monoclonal anti-myc antibody.

Figure 2. DNA minigene vaccine pHI-FLK_{400} and the DNA vaccine encoding the entire FLK-1 gene exhibit similar antitumor effects. Groups of mice (n=8) were immunized 3 times at 1wk intervals with attenuated *Salmonella typhimurium* harboring the vectors indicated. Mice were challenged 2 wk after the last immunization i.v. with $2x10^5$ EO771 breast carcinoma cells. Mice were sacrificed 21 days later and lung weight assessed. (A) Antitumor efficacy induced by minigene vaccine pHI-Db. (B) The antitumor efficacies were compared between vaccines encoding the entire FLK-1 based gene or minigene and signal peptide.

Figure 3. An H-$2D^b$ restricted FLK_{400}-specific response is induced by the pHI-D^b DNA minigene vaccine. Groups of C57BL/6 mice (n=4) were immunized 3 times at 1 wk intervals with attenuated *Salmonella typhimurium* harboring the vectors indicated. Two wk after the last

immunization, mice were sacrificed. (A) ELISPOT assays were performed on splenocytes isolated by using no stimulator (empty bars) or synthetic peptides FLK_{94} (25 □g/ml, shaded bars), FLK_{400} (10 □g /ml, solid bars), or FLK_{1210} (25 □g/ml, striped bars) as stimulators. (B-D) Isolated splenocytes were stimulated with FLK_{400} (B), FLK_{94} (C) or FLK_{1210} (D) peptides for 5 d. Thereafter cytotoxicity assays were performed with MS1 (solid diamonds, ♦) or RM9 (empty circles, ○) as target cells. (E-G) Splenocytes isolated from pHI-Db vaccinated mice were stimulated with FLK_{400} (E), FLK_{94} (F) or FLK_{1210} (G) peptides for 7 d, and restimulated twice weekly with irradiated FLK_{94} (E)-, FLK_{400} (F)- or FLK_{1210} (G)-loaded splenocytes from normal C57BL/6 mice. Thereafter cytotoxicity assays were performed with MS1 (solid diamonds, ♦) or RM9 (empty circles, ○) as target cells.

Figure 4. DNA vaccine encoding full length FLK-1 induces FLK_{400}-specific responses. (A-B) ELISPOT assays performed with splenocytes from pFLK-1 vaccinated mice: (A) freshly isolated splenocytes. Stimulator peptides include FLK_{94} (gray bar), FLK_{400} (black bar), FLK_{1210} (striped bar) and control with no peptide (empty bar). (B) Splenocytes were stimulated *in vitro* for 5d with peptides indicated as 'primary stimulators', then used in ELISPOT assays. Stimulators used in such ELISPOT assays are either unloaded (empty bars), FLK_{94}- (lighter solid bars), FLK_{400}- (darker solid bars), or FLK_{1210}- (striped bars) loaded splenocytes from normal C57BL/6 mice. Splenocytes from pFLK-1 (C), pHI-Db (D), pCMV (E) and pHI (F) groups were stimulated with irradiated MS1 cells for 5 d, and cytotoxicity assays were performed against unloaded (empty triangles, Δ) or FLK_{400}-loaded (solid squares, ■) EO771 breast cancer target cells.

Figure 5. Suppression of angiogenesis. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by

fluorimetry or confocal microscopy, respectively, using FITC-labeled isolectin B4. (A) Representative Matrigel plugs were examined by confocal microscopy (Magnification: x200). Arrows indicate borders of the Matrigel plug. Matrigel was implanted into mice vaccinated with either empty vector or pHI-Db. (B) The average fluorescence eluted from Matrigel plugs of each group of mice is depicted by bar graphs (n=4; mean + SD). Comparison of control with treatment groups of mice indicated statistical significance ($P<0.02$).

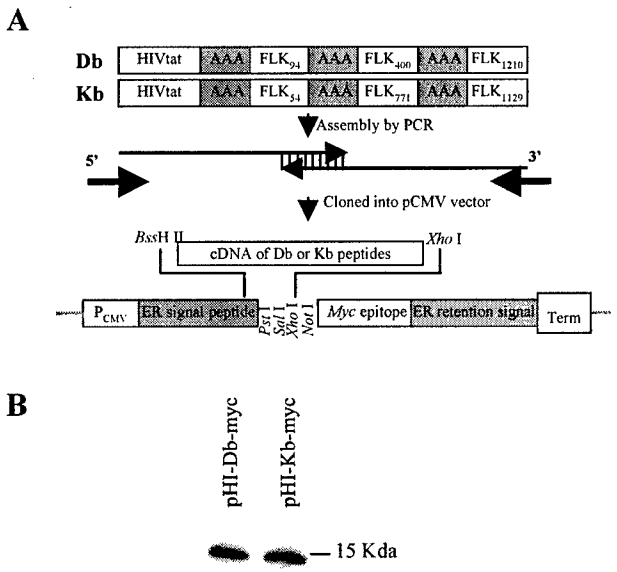
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Fig 1

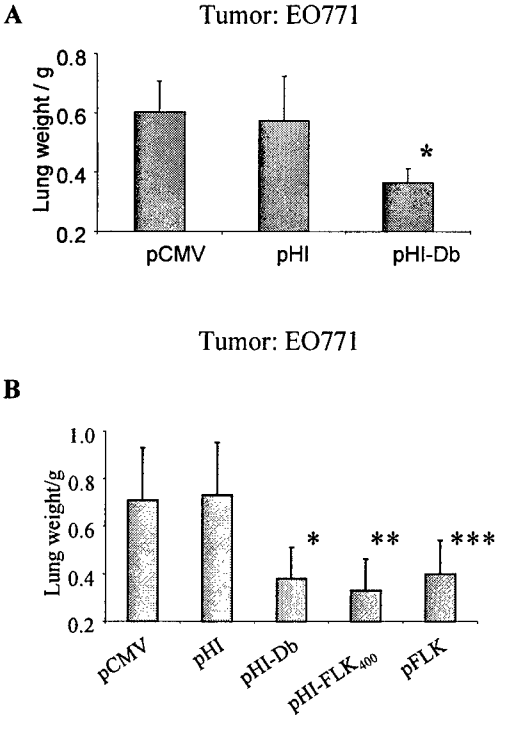
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Fig 2

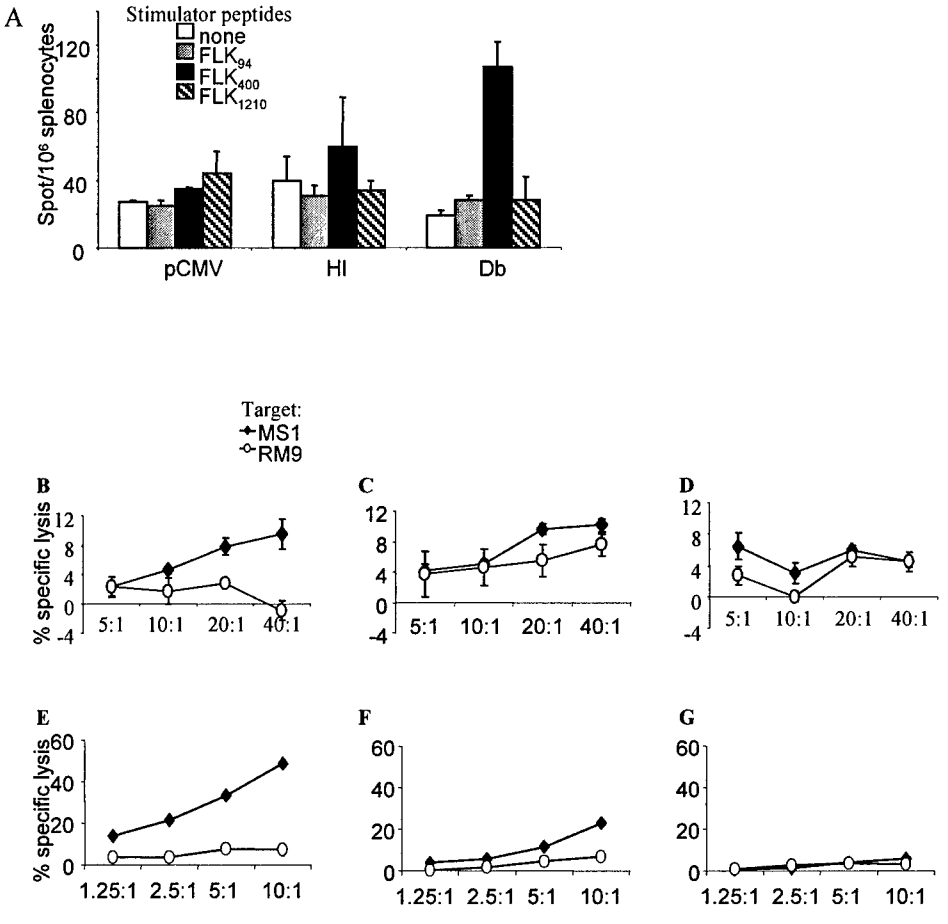
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Fig 3

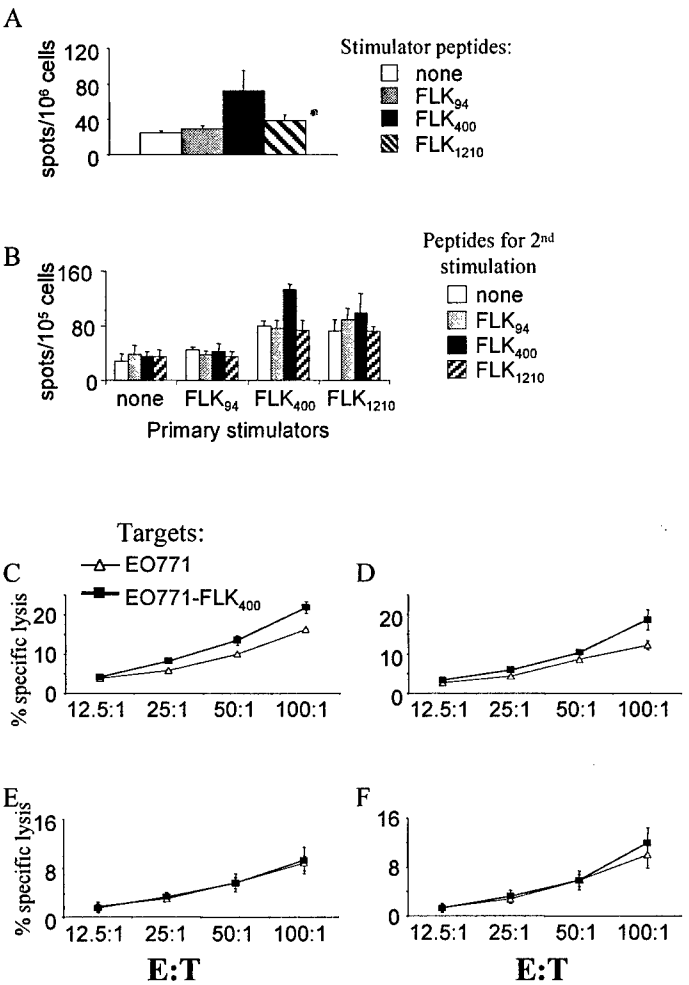
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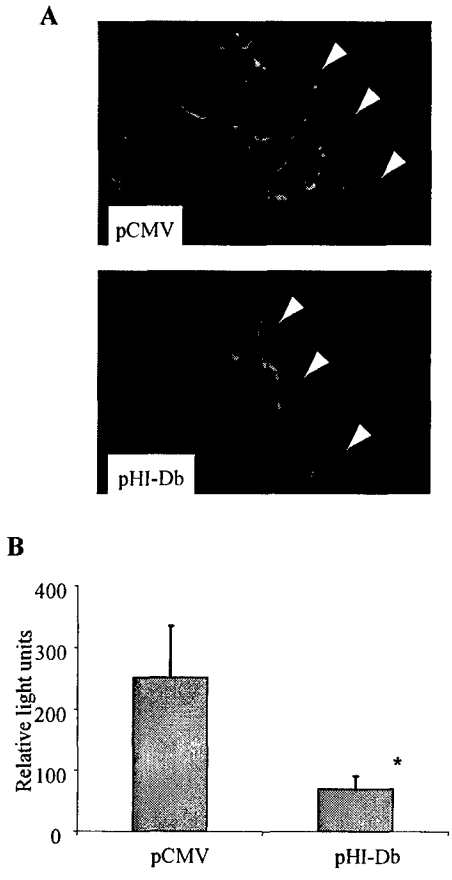
Fig 4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pCMV
pHI-Db

Fig 5

A DNA vaccine targeting Fos-related antigen 1 enhanced by IL-18 induces long-lived T cell memory against tumor recurrence

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Running Title: Memory T cell response against breast and colon cancer

Key Word: DNA vaccine; Fra-1; T cell memory; Interleukin 18; Non-lymphoid tissue

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Abstract

A novel vaccination strategy induced specific $CD8^+$ T cell-mediated immunity that eradicated spontaneous and experimental pulmonary cancer metastases in syngeneic mice and was also effective in a therapeutic setting of established breast cancer metastases. This was achieved by targeting transcription factor Fos-related antigen 1(Fra-1), overexpressed by many tumor cells, with an ubiquitinated DNA vaccine against Fra-1, co-expressing secretory IL-18. Insight into the immunological mechanisms involved was provided by adoptive transfer of T lymphocytes from successfully immunized BALB/c mice to syngeneic SCID mice. Specifically, long-lived T memory cells were maintained dormant in non-lymphoid tissues by IL-18 in the absence of tumor antigen. Importantly, a second tumor cell challenge of these SCID mice restored both, robust tumor-specific cytotoxicity and long-lived T cell memory, capable of eradicating established pulmonary cancer metastases, suggesting that this vaccine could be effective against tumor recurrence.

Introduction

The development of long-lived $CD8^+$ T cell memory is a major goal of vaccination against tumors since it can provide continuous protection against their further dissemination and recurrence. In fact, successful protection against tumors critically depends on both, an increased number of tumor antigen-specific $CD8^+$ T cells in an immune host and the distinct capability of CD8 T memory cells to proliferate, secrete inflammatory anti-tumor cytokines and repeatedly kill recurring tumor cells more effectively than naïve $CD8^+$ T cells. Consequently, several ongoing efforts focus on the development of DNA-based cancer vaccines capable of inducing long-lasting immunological memory responses endowed with specificity and a high potential to repeatedly kill tumor cells. Indeed, the establishment and continued long-term maintenance of immunological memory has been the very key to successful tumor protective vaccination strategies. (1-3)

Transcription factor Fos-related antigen 1(Fra-1), which is overexpressed by many human and mouse epithelial carcinoma cells (4-7), is involved in progression of various breast cancer cell types (8;9), and thus represents a relevant, potential target for a breast carcinoma vaccine. Indeed, we previously demonstrated that an oral DNA vaccine encoding murine Fra-1, co-expressing secretory murine interleukin18 (mIL-18), induced an effective cellular immune response capable of eradicating established D2F2 breast cancer metastases in syngeneic BALB/c mice (10). IL-18 is a well-known multi-functional cytokine that was co-expressed in our DNA vaccine to enhance tumor antigen presentation by DCs, and to maintain an antitumor immune response. IL-18 was originally believed to elicit cytokine production by T and/or NK cells and to induce their proliferation and cytolytic activity, similar to an IFN-γ-inducing factor (11). The anti-tumor activity of IL-18 is now considered to be primarily mediated by T-and

NK-cell activation and by enhancing cellar immune mechanisms via up-regulation of MHC class I antigen expression, favoring the differentiation of $CD4^+$ helper T cells toward the T helper 1 (Th1) subtype. In turn, Th1 cells secrete proinflammatory cytokines IL-2 and IFN-γ, which facilitate the proliferation and/or activation of $CD8^+$ CTLs, NK cells, and macrophages, all of which can contribute to tumor regression (11;12). In addition, IL-18 is an important mediator of memory $CD8^+$ T cell proliferation and activation via bystander activation. This process was extensively studied by Sprent and colleagues (13;14), who demonstrated that administration of innate immune activators induces proliferation of memory $CD8^+$ T cell through a mechanism involving type I IFN, IL-12, IL-15 and IL-18, respectively.

Here, we extended our prior studies on a Fra-1 based DNA vaccine co-expressing IL-18 in two breast tumor models as well as a non-small cell lung carcinoma model by investigating potential working mechanisms of this vaccine, focusing particularly on the generation, function and long-term survival of $CD8^+$ memory T cells in tumor models of syngeneic BALB/c and SCID mice after adoptive transfer of T cells from successfully vaccinated mice. We also focused on $CD8^+$ T cells that could remain dormant at high frequency in non-lymphoid tissue after successful vaccination, because their ultimate presence in the periphery is important for eliciting resistance against secondary tumor cell challenges. These questions were addressed with a polyubiquitinated DNA vaccine encoding Fra-1, co-transformed with secretory murine IL-18 and carried by attenuated *Salmonella typhimurium*, which proved capable of inducing a long-lived $CD8^+$ T cell response that eradicated recurring D2F2 breast cancer metastases in syngeneic BALB/c mice.

Materials and Methods

Animals, Bacterial Strains and Cell Lines. Female BALB/c and C57BL/6 mice, 6-8 wk of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. Female SCID mice were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice were maintained under specific pathogen-free conditions and used for experiments when 7 wk old. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The double attenuated *S. typhimurium* strain RE88 (*aroA*$^-$ *dam*$^-$) was obtained from Remedyne Corporation (Santa Barbara, CA). The murine D2F2 breast cancer cell line was kindly provided by Dr. Wei-Zen Wei. (Karmanos Cancer Institute, Detroit, MI) The murine D121 non-small cell lung carcinoma cells were a gift from Dr. L. Eisenbach. (Weizmann Institute of Science, Rehovot, Israel) and the murine 4T1 breast carcinoma was kindly provided by Dr. Suzanne Ostrand-Rosenberg.(University of Maryland, Baltimore, MD)

RT-PCR, Western blotting and Immunohistochemistry. *RT-PCR*: Total RNA was extracted with the Rneasy mini Kit or Rneasy tissue Kit (Qiagen, Valenica, CA) from 3×10^6 tumor cells of various origin: breast carcinoma cells D2F2, 4T1 and 4T07; colon carcinoma cells CT26; prostate carcinoma cells RM2; non-small cell lung carcinoma cells D121 and normal tissues from mouse spleen, liver, lung and bone marrow. Reverse transcription was performed with 1μg of total RNA followed by PCR with the appropriate oligonucleotides. The following primers were used: ATGTACCGAGACTACGGGGAA (forward); and TCACAAAGCCAGGAGTGTAGG (reverse). The PCR was cycled 30 times at 52°C annealing temperature and quantities of RNA and PCR were monitored for glyceraldehyde-3-phosphate dehydrogenase resulting in an 821bp fragment. *Western blots*: Fra-1 protein expression was established in the above mentioned array of tumor cell lines and murine tissues. Western blot analysis was done with the total protein from cell lysate homogenates,

using a polyclonal primary rabbit anti-murine Fra-1 Ab and anti-murine β-actin Ab as a loading control (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Specific protein was detected with a goat anti-rabbit-HRP conjugated IgG Ab (Biorad, Richmond, CA). *Immunohistochemistry:* D2F2 tumor tissues were cut into sections and exposed to air until completely dry, fixed in cold acetone at -20 °C for 10 min and stained for Fra-1 with the DAKO immunostaining system (DAKO, Carpinteria, CA), using rabbit anti murine Fra-1 Ab (Santa Cruz Biotechnology Inc., Santa Cruz, CA), diluted 1:1000 or negative control reagents. This was followed by incubation at 4 °C overnight. After 3 washes with PBS, a HRP conjugated goat anti-mouse secondary Ab (DAKO) was used, and slides mounted with DAKO Faramount (DAKO). Cells were visualized microscopically, and images captured with a Nikon digital camera (Japan) linked to a workstation with Adobe photoshop^{TM} software (Adobe System Incoporated, San Jose, CA)

Vector construction and protein expression. Two constructs were made based on the vectors pcDNA3.1/Zero, and pSecTag2/Hygro (Invitrogen, San Diego, CA), respectively. The first construct, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fos-related-antigen-1 (Fra-1). The second construct, pIL-18, contained mIL-18 with an Igκ leader sequence for secretion purposes. The empty vector served as a control. Protein expression of Fra-1 and IL-18 were demonstrated by Western blotting with a polyclonal rabbit anti-murine Fra-1 Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a monoclonal anti-mouse IL-18 Ab (R & D Systems, Minneapolis, MN), respectively. IL-18 protein was detected in both cell lysates and culture supernatants.

Transformation and Expression of S. typhimurium with DNA Vaccine Plasmids. Attenuated *Salmonella typhimurium* (dam$^-$; aroA$^-$) were transduced with DNA vaccine plasmids by electroporation. Briefly, a single colony of bacteria was inoculated into 3 ml of LB medium,

harvested during mid-log growth phase, and washed twice with ice-cold water. Freshly prepared bacteria (1×10^8) were then mixed with plasmid DNA (2 μg) on ice in a 0.2-cm cuvette and electroporated at 2.5 KV, 25 μF and 200 Ω. The bacteria were transformed with the following plasmids: empty vector; pUb-Fra-1; pIL-18; or both pUb-Fra-1 and pIL-18 combined, indicated as pUb-Fra-1/pIL-18. After electroporation, resistant colonies harboring the DNA vaccine gene(s) were cultured and stored at -70° C after confirmation of their coding sequence.

Immunization and Tumor Cell Challenge. *Prophylactic model*: BALB/c mice or C57BL/6 mice were divided into four experimental groups (n=8) and immunized three times at 2 wk intervals by gavage with 100 μl 4.5% sodium bicarbonate containing 1×10^8 doubly mutated *S. typhimurium* harboring either empty vector; pUb-Fra-1; pIL-18; or pUb-Fra-1/pIL-18. All mice were challenged by i.v. injection with 5×10^5 D2F2 cells(BALB/c) or 2×10^5 D121 cells (C57BL/6) or fat-pad injection with 7×10^3 4T1 cells(BALB/c), 1 wk after the last immunization, to induce either experimental or spontaneous pulmonary metastases. The survival rate of mice, lung weight and metastatic scores in experimental or control groups were observed. Two months after the first tumor cell challenge, the surviving mice from the treatment groups in only the D2F2 model were divided into two groups; one was re-immunized with pUb-Fra-1/pIL-18 and the other group received no re-immunization. All mice were re-challenged with 0.5×10^6 D2F2 cells 1 wk after re-immunization and sacrificed at either 2 or 8 wk after the first challenge or 1 wk after re-challenge with tumor cells to determine the proliferation of $CD8^+$ T cells and IFN-γ release. *Therapeutic model:* BALB/c mice were divided into four experimental groups (n=8) and injected i.v. with 0.5×10^6 D2F2 cells on day 0 and then immunized with DNA vaccine three times as described above. The experiment was terminated on day 28 to observe mouse lung weights.

Adoptive transfer of lymphocytes. BALB/c mice which served as donors of tumor-specific $CD8^+$ T cells for adoptive transfer experiments were those animals who were previously successfully immunized 3 times at 2 wk intervals with the pUb-Fra-1/pIL-18 vaccine. These animals were then challenged with 0.5×10^6 D2F2 tumor cells i.v. 1 wk later and remained tumor free 2 months thereafter. Mice comprising the control group were immunized only with empty vector. The animals in all treatment groups were sacrificed 2 d after being re-immunized with the pUb-Fra-1/pIL-18 vaccine or after receiving no re-immunization. Lymphocytes were harvested via cannulation of the spleen and separated by Ficoll/hypaque gradient centrifugation (600×g, 20min). Naïve syngeneic SCID mice were reconstituted with a total of 4.5×10^7 lymphocytes by i.v. injection of 1.5×10^7 lymphocytes each, on days 0, 2, 4. Their fate was then followed by flow cytometric analyses on days 7, 14 and 30, with anti-CD8 and anti-CD3 Abs, respectively. After 2 d, individual groups of mice were challenged by i.v. injection of 0.5×10^6 D2F2 cells to initiate experimental pulmonary metastases. Tumor specific cytotoxicity and IFN-γ release, were determined 3, 7 and 30 d after tumor cell challenge and the survival rate of these SCID mice was observed.

Preparation of lymphocytes from nonlymphoid tissue. The lung vascular bed was flushed with 10ml chilled Hank's balanced salt solution (HBSS, GiBCO) introduced via cannulation of the right ventricle. Lymphocytes were incubated for 60 min at 37°C in a solution of enzymes i.e. 125u/ml collagenase (Gibco-BRL), 60u/ml each of Dnase I and 60u/ml of hyaluronidase (Sigma, St,Louis MO). The cell suspension was then layered over a lymphocyte-M (Cedarlane Laboratories, Horby, Canada) density gradient (15), centrifuged at 600g for 20 min at 25°C, and lymphocytes washed 2-3 times prior to further processing. Blood was flushed from livers by injecting 5 ml of RPMI-1640 through the portal vein. Leukocytes from the liver were then

isolated by crushing this organ in a tissue grinder, followed by incubation with the above enzyme solution and collection of the leukocyte layer from a Metrizamide (Sigma-Aldrich, St. Lois, HO) density gradient. Contaminating erythrocytes were removed from the leukocyte preparations by treatment with ACK lysis buffer (Cambrex Bio Science Walkersville, Inc., Walkersville MD).

In vitro depletion $CD4^+$ or $CD8^+$ T cells. The depletion of $CD4^+$ or $CD8^+$ T cell in vitro was performed as previously described (16). Briefly, splenocytes were isolated from C57BL/6 mice after vaccinations with experimental or control DNA vaccines, 2 wk after challenge with D121 tumor cells. $CD4^+$ T cells depletion was accomplished with $10ug/10^7$ splenocytes of anti-CD4 (derived from hybridoma GK1.5), and $CD8^+$ T cells were depleted with anti-CD8 Ab (derived from hybridoma 2.43) for 30min at 37 °C. Then rabbit serum complement (1:6) was added with 1 ml diluted complement to 10^7 cells /ml. Cells were incubated for 30 min at 37 °C, washed and re-suspended for the CTL assay. All antibodies were purchased from National Cell Culture Center, Minneapolis, MN. Rabbit serum complement was obtained from Serotec. Inc. Raleigh, N.C.

Cytotoxicity Assay. Cytotoxicity was measured and calculated by a standard ^{51}Cr-release assay. Briefly, in the D2F2 tumor model splenocytes were harvested from BALB/c SCID/SDCI mice at 3, 7 and 30 days after challenge with 5×10^5 D2F2 breast carcinoma cells after passive transfer of lymphocytes. In the D121 lung tumor model, splenocytes were obtained from C57BL/6 mice 2 wk after challenge with 2×10^5 D121 tumor cells following either $CD4^+$ or $CD8^+$ T cell depletion in vitro. These cells were then stimulated *in vitro* by irradiated (1000 Gy) D2F2 cells or D121 cells for 4 days at 37° C in complete T-STIM culture medium (Becton Dickinson, Bedford, MA) containing 10% FBS and recombinant IL-2 at 20 U/ml (PeproTech, Rocky Hill,NJ). These D2F2 or D121 target cells were then labeled with ^{51}Cr for 2 h at 37° C

and incubated with effector cells at various effector-to-target cell ratios at 37°C for 4 h. The percentage of specific target cell lysis was calculated by the formula[(E-S)/(T-S)]×100, where E is the average experimental release, S the average spontaneous release, and T the average total release.

Flow cytometry. Activation markers of T cells were measured by two-color flow cytometric analysis with a BD Biosciences FACScalibur. T cell markers were determined by staining freshly isolated lymphocytes from successfully vaccinated mice or from passively transferred SCID mice with anti-CD8 Abs in combination with FITC –conjugated anti-CD3 Ab. Memory $CD8^+$ T cells bearing high levels of $CD44^{high}$ and $CD122^+$ (IL-2Rβ) were quantified by three-color flow analysis. Splenocytes were isolated from successfully vaccinated BALB/c mice or from passively transferred SCID mice and then stained with anti-CD8-Cy-chrome, anti-CD122-PE and anti-CD44-FITS Abs, followed by FACS analyses. All antibodies were purchased from PharMingen, San Diego, CA. IL-2 release at the intracellular level were determined in lymphocyte of Peyer's Patches obtained 3 days after one time immunization and stained with APC- anti-CD4 or CD8 and combined with FITS-anti-CD69. Cell were fixed, permeabilized and subsequently stained with PE-labeled anti-IL-2 Abs to detect the intracellular expression of IL-2.

ELISPOT assay. ELISPOT assays were performed to measure single cell release of INF-γ. Splenocytes were collected 2 wk after D121 tumor cell challenge from all experimental groups of C57BL/6 mice or 2, 7 and 30 d after lymphocyte transfer to SCID mice (only in the D2F2 tumor model), and splenocytes from control mice immunized only with the empty vector. After lysis of red blood cells with ACK lysis buffer, splenocytes were re-suspended at a final concentration of 1×10^7/ml (D121 tumor model) or 2×10^6 /ml (D2F2 tumor model), and 100 ul of this suspension were cultured for 24 h in complete T cell medium with or without 100ul

irradiated (1000Gy) D121 cells (1×10^5/ml) or D2F2 cells (1×10^4/ml). The assay was performed according to instructions provided by the manufacturer (BD Bioscience). Plates were read by immunospot®ScAnalysis and digitalized images were analyzed for areas in which color density exceeded background by an amount based on a comparison with experimental wells.

Statistical Analysis. The statistical significance of differential finding between experimental groups and controls was determined by Student's *t* test, Findings were regarding as significant, if two tailed *P* values were <0.05.

Results

Differential Expression of Fra-1 in tumor cell lines and normal mouse tissues. To study the distribution and expression of the Fra-1 antigen in mouse tumor models, we examined its differential expression in normal and mouse tumor tissues by analyzing expression of mRNA with RT-PCR in normal tissues of spleen, liver, lungs and bone marrow and in breast tumor cell lines D2F2, 4T1, 4T07, prostate carcinoma RM2, non-small cell lung carcinoma D121 and CT26 colon carcinoma cells. Expression of mRNA levels of Fra-1 was markedly increased in all of these tumor cell lines but was detectable only at very low levels in all normal tissues (Fig. 1A). This differential expression of Fra-1 was confirmed at the protein level by Western blotting, revealing high expression in D2F2 cells and somewhat lower expressions in RM2 and CT-26 cells. In contrast, Fra-1 protein level was uniformly expressed at very much lower levels in all of the normal murine tissues examined (Fig 1B). Furthermore, immunohistochemical analysis indicated strong Fra-1 expression in D2F2 breast cancer tissue when paraffin embedded sections were stained with anti-Fra-1 antibody (Fig 1C-I, III) when compared with negative control sections stained without the primary anti-Fra-1 antibody (Fig.1C-II, IV).

Fra-1/IL-18 base DNA vaccine induces effective anti-tumor immunity. We proved our hypothesis that an orally administered DNA vaccine encoding murine Ub-Fra-1 and secretory IL-18, carried by attenuated *S. typhimurium* , can induce an effective anti-tumor immune response. We found an increase in lifespan of BALB/c mice (n=8) vaccinated as described above, and challenged 2wks later by i.v. injection of a lethal dose (5×10^5/ml) of D2F2 breast carcinoma cells. The lifespan of 62% of successfully vaccinated BALB/c mice (5/8) tripled in the absence of any detectable tumor growth up to 98 days after tumor cell challenge (Fig2. A-a)

Vaccination reduces growth of established metastases. Marked inhibition of growth of established metastases was observed in C57BL/6 mice challenged by i.v. injection of D121 non-small cell lung carcinoma cells 2wks after the third vaccination with the Fra-1/IL-18 based vaccine as described above. In contrast, animals vaccinated with only the empty vector carried by the attenuated bacteria, revealed uniformly rapid metastatic pulmonary tumor growth of D121 non small cell lung carcinoma (Fig2. A-b). Our vaccine was also effective in a therapeutic setting. This was shown by an initial i.v. injection of BALB/c mice (n=8) with D2F2 breast carcinoma cells and vaccination of these mice 5 d thereafter with our Fra-1/IL-18 vaccine when these mice had established pulmonary metastases, and by collecting their lungs 28 d later. All such treated mice showed lower lung weights and markedly reduced tumor burden, whereas all control animal treated with the empty vector revealed much increased lung weights and tumor burden. (Fig2.B)

Protection against spontaneous pulmonary metastases. We noted a marked reduction in dissemination of spontaneous pulmonary metastases of 4T1 breast carcinoma cells after three immunizations with the Fra-1/IL-18 based DNA vaccine. This became evident 28 days after surgical excision of fat-pads bearing primary 4T1 breast carcinoma and as confirmed by visual

examination of the lungs of these animals for metastases, which established their metastatic score (Fig2. A-c)

$CD8^+$ T cells are responsible for the antitumor response. Evidence for an activated T cell immune response was indicated by three lines of evidence. First, we found that only the vaccine encoding pUb-Fra-1/pIL-18 proved highly effective in markedly up-regulating IL-2 expression on $CD69^+$, $CD4^+$ or $CD8^+$ activated T cells during T cell priming (Fig3.A). Second, only lymphocytes isolated from mice immunized with this vaccine were effective in specifically killing D121 breast cancer cells in vitro at different effector-t0-target cell ratios. In contrast, lymphocytes isolated from vaccinated mice which were thereafter depleted of $CD8^+$ T cell in vitro failed to induce cytotoxic killing of D121 tumor target cells. However, in vitro depletion of $CD4^+$T cell did not abrogate cytotoxic killing of these same tumor target cells. (Fig 3B) The same results were obtained in the D2F2 breast carcinoma model in BALB/c mice (data not shown). Third, release of IFN-γ from T cells, a well-known indication of T cell activation in secondary lymphoid tissues, was found at the single cell level by ELISPOT (Fig. 3C) only after vaccination with the pUb-Fra-1/pIL-18 plasmid. In fact, subsequent challenge with tumor cells induced a dramatic increase in IFN-γ release when compared to that of splenocytes from control mice. Taken together, these data suggest that the activation of T cells was specific for Fra-1.

Activation of Specific T cells in lymphoid tissue is followed by migration to nonlymphoid tissue. Interactions between IL-18 and active Th1 cells are believed to be critical for achieving both, optimal Ag-specific T cell responses in lymphoid tissues and activated T cell migration to non-lymphoid tissues in the local tumor microenvironment. To prove this contention in our models, we analyzed $CD8^+$ T cell in both lymphoid and non-lymphoid tissues. The vaccine encoding pUb-Fra-1/pIL-18 substantially up-regulated the $CD8^+$ T cell population 2

wk after challenge with D2F2 tumor cells in both lymphoid tissues (spleen) as well as in blood and nonlymphoid tissues, especially in local lung tumor tissues (Fig. 4A I). Eight weeks after tumor cells challenge, the level of $CD8^+$ T cells in all these tissues declined and, in fact, this decline was more rapid in lymphoid tissues (spleen) than in nonlymphoid tissues (lungs) (Fig. 4A II). However, when 8 wk after the initial tumor cell challenge, all surviving mice were re-challenged with D2F2 cells, $CD8^+$ T cells population were again dramatically upregulated and these same cells increased even more rapidly in lung tissue than in lymphoid tissues (Fig. 4A III). Furthermore, ELISPOT assay indicated INF-γ release from these same lymphocytes and T cell activation was confirmed by release of proinflammatory cytokine INF-γ which increased in both lymphoid and nonlymphoid tissues 2 wk after the first tumor cell challenge (Fig. 4B I). However, 8 wk after the initial tumor cell challenge, INF-γ release decreased (Fig.4A II) but then increased again dramatically after re-challenge with D2F2 tumor cells, especially in lung lymphocytes. (Fig.4A III).

A Specific memory T cell response is induced and maintained in the absence of tumor antigen. We tested the hypothesis that $CD8^+$ T cells, adoptively transferred from successfully immunized mice to syngeneic SCID mice, and parked there for 7 or 30d, could maintain effective and long-lived memory in the absence of both tumor Ag and naïve T cells. To this end, SCID mice were adoptively transferred with lymphocytes that were harvested from successfully immunized mice that had remained tumor free for at least 98 d after the initial tumor cell challenge, and were then subjected to either re-immunization with the same DNA vaccine or to no re-immunization. The data depicted in Fig. 5A indicate that the lifespan of 75% (6/8) in the re-immunized group of mice and in 62% (5/8) in the non-re-immunized group of SCID mice was tripled in the absence of any detectable tumor growth up to 56 d after tumor cell challenge.

Importantly, the continuous presence of tumor Ag was not required in order to maintain long-lived $CD8^+$ T cell memory among $CD8^+$ T cells that were adoptively transferred into syngeneic SCID mice (Fig.5A.). Furthermore, we determined the fate of $CD8^+$ T effector cells in the absence of tumor antigen by adoptive transfer of lymphocytes from immunized BALB/c mice into SCID mice. Thus when these animals' splenocytes were subjected to FACS analysis to detect the presence of $CD8^+$ T cells, there was a continuous decrease in the number of these cells for 30 d suggesting that the majority of these T effector cells gradually apoptosed. (Fig.5B),

Rapid turnover of memory $CD8^+$ T cells after vaccination and repeated tumor cell challenges. We determined that vaccination with the pUb-Fra-1/IL-18 construct, followed 2 wk thereafter by a D2F2 tumor cell challenge, leads to a rapid turnover of $CD8^+$ T memory cells. This was indicated by upregulated expression of $CD8^+$, $CD44^{high}$, $CD122^+$ memory T cell markers at different time points in both lymphoid and non-lymphoid tissues (Fig. 6A). Thus, turnover of these memory T cells occurred just 24 h after tumor cell challenge and reached a peak at 72 h (Fig.6B). Importantly, we could also verify that these putative, specific $CD8^+$ memory T cells can also effectively recognize a second challenge of D2F2 tumor cells. If fact, we not only detected increased expression of $CD8^+$, $CD44^{high}$, $CD122^+$ memory T cells in both lymphoid and non-lymphoid tissues 56 d after the first tumor cell challenge in syngeneic BALB/c mice (Fig. 6C-I), but also found the same upregulation of these $CD8^+$ memory T cell markers when these very same lymphocytes were adoptively transferred to SCID mice that were subjected 1 wk thereafter to a D2F2 tumor cell challenge (Fig.6C-II). Moreover, the $CD8^+$, $CD44^{high}$, $CD122^+$ memory T cell expression was more pronounced in local lung tumor tissues than in the spleen. Taken together, these data demonstrate that specific $CD8^+$ T memory cells

rapidly turned over after a successful immunization with our vaccine and could again respond effectively to the next tumor cell challenge.

Cytotoxic T cell response to a secondary D2F2 breast cancer cell challenge in SCID mice. We could demonstrate that after vaccination with pUb-Fra-1/IL-18 and subsequent tumor cell challenges, activated T cell can be successfully transferred adoptively to SCID mice where they also respond to a secondary challenge of D2F2 tumor cells. Specifically, SCID mice were challenged with D2F2 tumor cells after adoptive transfer of splenocytes from successfully immunized BALB/c mice. The data depicted in Fig.7 indicate that putative $CD8^+$ T memory cells that had been parked for up to 30 d in SCID mice did effectively recognize a secondary challenge of D2F2 tumor cells. Furthermore, we observed that these activated $CD8^+$ T cells released increased amounts of INF-γ (Fig. 7A) and were highly effective in cytotoxic killing of D2F2 breast cancer cells *in vitro* at different effector-to-target cell ratios (Fig. 7B).

Discussion

Fos-related antigen-1 (Fra-1), a transcription factor of the AP-1 family, was shown previously to be involved in tumor cell progression and to be overexpressed in many human and murine tumor tissues (4-7). Here, we also provide evidence that Fra-1 is highly expressed in a variety of tumor cell lines at both the mRNA and protein levels. This finding suggests that Fra-1 could be linked to the malignancy of murine tumor cells and provides a potential target for immunotherapy of cancer especially breast cancer cells. In fact, our previous work demonstrated that a DNA vaccine targeting Fra-1 and co-expressing IL-18 could induce an effective cellular immune response, which led to the eradication of established D2F2 breast cancer metastases in syngeneic BALB/c mice (10). Here, we further extended our prior studies. Thus we

hypothesized that immunization with a DNA vaccine encoding murine Fra-1, fused to polyubiquitin and modified by co-transformation with a gene encoding secretory murine IL-18, will work effectively in two different breast carcinoma models as well as in a non-small cell lung carcinoma model and induce strong anti-tumor activity in syngeneic mice which can be maintained as a long-lived specific immune response against breast cancer cells. Meanwhile, IL-18 enhanced immune responses by activating T and NK cells while up-regulating MHC class I antigen expression and assisting the differentiation of $CD4^+$ T cells toward the Th1 subtype. Additionally, the effective generation and maturation of $CD8^+$ CTLs should result in an effective Th1 type immune response. Proof for this hypothesis was established by the induction of anti-tumor immune responses in three mouse tumor models, two of breast carcinoma (D2F2, 4T1) and one non-small cell lung carcinoma (D121), both against primary tumors and their respective spontaneous and experimental pulmonary metastases. Our vaccine was also effective in a therapeutic setting of established pulmonary metastases. Three lines of evidence indicated that the effector cells responsible for this tumor protective immunity were primarily activated $CD8^+$ T cells. First, $CD8^+$ T cells isolated from splenocytes of specifically vaccinated mice specifically killed D2F2 and D121 target cells, respectively in *in vitro* cytotoxicity assays. Second, the DNA vaccine did indeed activate $CD8^+$ T cells, because such cells isolated from splenocytes of successfully vaccinated mice, secreted the Th1 pro-inflammatory cytokine IFN-γ, and $CD8^+$T cell populations were markedly up-regulated. Third, $CD8^+$ cells were activated both in lymphoid and non-lymphoid tissues, especially those located in tumor tissues of lung and liver. The mechanism of tumor protection is thought to depend on $CD4^+$ T cells and is potentially mediated by helper T cells, associated with effector functions and/or cytokine release which combine to break immunological tolerance to tumor antigen. Our finding further supports this contention

that both $CD4^+$ T cells and IL-2 release from these cells in the Peyer's Patch was markedly up regulated after one time vaccination.

The establishment and long-term maintenance of immunological memory is a requirement for all protective vaccination strategies. Here, we demonstrated that $CD8^+$ cells isolated from splenocytes of successfully vaccinated BALB/c mice, when adoptively transferred to syngeneic SCID mice, maintained sufficient memory to markedly suppress dissemination and growth of a lethal challenge of D2F2 breast cancer cells. This finding was further supported by the markedly increased release of IFN-γ and $CD8^+$ T cell cytotoxicity. Importantly, we found strong, local expression of $CD8^+$ T cells in tumor tissues, indicating that a $CD8^+$ T cell response does not only occur in lymphoid tissues after successful vaccination, but that these T cells can also migrate to non-lymphoid tissues. This occurs particularly in the tumor microenvironment where $CD8^+$ T cells react against antigen-positive tumor cells. It is indeed relevant that some of these $CD8^+$ T cells were subsequently found as long-lived memory T cells that were ready to respond to the next stimulation upon re-encounter with the same tumor antigen. In this regard, it is well known that optimal T cell activation results in clonal expansion, re-distribution into-non-lymphoid tissues and subsequent formation of memory (17;18). It was also postulated that the immune response to foreign antigen is not necessarily limited to secondary lymphoid tissue. Importantly, non-lymphoid sites are essential for activated T cell function and subsequent immunosurveillance. Most early studies of tumor antigen-specific T cell responses were limited to analyses of lymph nodes, spleen and blood. However, non-lymphoid tissues differ from organized secondary lymphoid organs in both, the quality and quantity of cytokines, lymphocytes as well as immune accessory cells (19;20). Importantly, we found that our DNA vaccine can induce protective anti-tumor immune responses, which not only occur in lymphoid

tissues but also in non-lymphoid tissue near tumor sites and that, in addition, antigen-specific $CD8^+$ T cells can also migrate to non-lymphoid tissues and remain there for long periods of time as dormant memory cells. Strikingly, lymphocytes isolated from non-lymphoid tissues, such as lungs in our study, exhibited a greater release of IFN-γ and contained a higher percentage of $CD8^+$ CTLs as well as memory $CD8^+$ T cells than their splenic counterparts. These results point to the existence of a population of extra-lymphoid effector memory T cells poised for an immediate response to tumor-associated antigen. In fact, recent studies indicated that certain cytokines could induce bystander proliferation *in vivo* by T cells with a memory phenotype such as Type I IFN, IFN-γ, IL-15, IL-12 and IL-18. Moreover, it was found that injection of IL-18 stimulated a strong increase in the BrdU labeling of memory phenotype $CD8^+$ T cells *in vitro*. Furthermore, IFN-γ, which is inducible by IL-18, is also capable of promoting the turnover of memory phenotype CD8+ T cells (21). Based on the finding that T cell proliferation induced by IL-12 and IL-18 was dependent on IFN-γ (21), we examined the effect of IL-18 on T cell turnover. Indeed, our results support the concept that co-expression of secretory IL-18 in our DNA vaccine induced the rapid turnover of $CD44^+$, $CD122^+$, $CD8^+$ T cells within 24 h after immunization and that such $CD8^+$ T memory cells can be maintained in lymphoid tissues as well as locally in lung tumor tissues. Interestingly, $CD8^+$ T cell found in the lungs of our vaccinated mice were able to proliferate and acquire strong IFN-γ releasing capabilities after Ag exposure *in vitro*. Consequently, it is reasonable to conclude that persistently activated T cells and memory $CD8^+$ T cells in the lung can play a key role in the cellular immune response against tumor metastases.

The role of persisting Ag in T cell memory cells and the requirement of such cells for chronic exposure to residual deposits of Ag for maintenance of $CD8^+$ T cell memory have been the

subject of much discussion and controversy (22-24). This is in contrast to $CD8^+$ effector T cells that absolutely do not require the presence of Ag. In fact, this is consistent with a decrease in adoptively transferred $CD8^+$ T cells observed in our studies in the absence of Ag. (Fig.5*a*). Indeed, a number of reports indicate that memory T cells survive poorly following adoptive transfer, unless accompanied by specific Ag (25-27). However, the contention that memory T cells require constant Ag stimulation has been challenged by reports demonstrating that $CD8^+$ memory cells can survive for prolonged periods of time after adoptive transfer in the absence of Ag (28-31). Our findings suggest that, at least for $CD8^+$ T cells, some memory T cells do not require continuous stimulation with Ag for survival.

In summary, we could demonstrate that an oral DNA vaccine, encoding Ub-Fra-1 and IL-18, carried by an attenuated strain of *S. typhimurium,* protected BALB/c mice against a lethal challenge of murine D2F2 and 4T1 breast cancer cells, and C57BL/6 mice against D121 lung carcinoma cell challenge. Moreover, this vaccine is capable of breaking T cell tolerance to a self-antigen and generates a long-lived memory T cell immune response against recurring breast cancer which could be maintained consistently in SCID mice in the absence of tumor antigen in both lymphoid and non-lymphoid organs.

Figure legends

Fig. 1. Expression of the murine Fra-1 molecule in normal mouse tissues and tumor cell lines. A.) RT-PCR analysis of Fra-1 gene expression by carcinoma cell lines D2F2, 4T1, 4T07 (breast), CT-26 (colon), RM2 (prostate) and D121 (non-small cell lung) as well as normal murine tissues from spleen, liver, lungs and bone marrow. Total RNA was extracted from cells growing at 70% confluence and from normal murine tissues. GAPDH was used as a control for total RNA loading. B.) Western blot analysis of Fra-1 protein expression in the above mentioned tumor cell lines and normal mouse tissues. Protein lysates were extracted from cells growing at 80% confluence. Homogenized normal tissues and β-actin were used as controls for protein loading. C.) Immunohistochemical analysis of Fra-1 in D2F2 breast cancer tissue. Paraffin imbedded sections from D2F2 breast cancer tissue samples were analyzed by immunohistochemistry using Ab against Fra-1 protein. Immunohistochemical staining of D2F2 breast cancer tissue with anti-Fra-1 Ab.(I, ×10 magnification; III, ×40 magnification) and, without using primary anti-Fra-1 antibody. (II, ×10 magnification; IV, ×40 magnification)

Fig. 2. Effect of protective immunity induced by the pUb-Fra-1/pIL-18 based DNA vaccine on different tumor metastases models. A.) *Prophylactic model:* Vaccination schedule designed for 3 immunizations at 2 wk intervals, followed by i.v. challenge with 0.5×10^6 D2F2 or 0.2×10^6 D121 tumor cells or fat pad injection with 0.7×10^4 4T1 tumor cells 1 wk after the last immunization. a.) The Kaplan-Meyer plot represents the survival of 8 mice in each of the treatment regimens (□) pUb-Fra-1, (△) pIL-18, (×) pUb-Fra-1/pIL-18 and control groups (◊). Surviving mice were tumor free unless otherwise stated. b.) Representative lung specimens of C57BL/6 mice (n=8) were obtained 4 wks after challenge with D121 non-small cell lung carcinoma cells. Bar graphs indicate average lung weight (g) in each group. Normal lung weight is approximately 0.2g. **, *P*

<0.001, P<0.05, P<0.05 compared to empty vector, pIL-18 or pFra-1, respectively. Experiments were repeated three times with similar result. c.) Representative lung specimens from BALB/c mice (n=8) challenged with 4T1 breast carcinoma cells by fat-pad injection 4 wk after removal of primary tumor. Tumor metastatic scores on lungs were established by estimating the% surface area covered by metatases as follow: 0= no metastases; 1=<20%; 2= 20-50% and 3=>50% are represented by individual symbols for each treatment group, and the short lines represent the average metastatic score of each group. **, *P <0.001, P<0.05, P<0.05* compared to empty vector, pIL-18 or pFra-1, respectively. B.) *Therapeutic model:* Groups of BALB/c mice (n=8) were initially injected i.v. with 0.2×10^6 D2F2 cells on day zero and then vaccinated with the DNA vaccine on days 5, 12 and 19, respectively. The experiment was terminated on day 28. Bar graphs indicate average lung weight (g) in each group. **, *P <0.001, P<0.05, P<0.05* compared to empty vector, pIL-18 or pFra-1, respectively.

Fig. 3. T cell activation by the pUb-Fra-1/pIL-18 based vaccine in the non-small cell lung carcinoma model. A.) *Up-regulated IL-2 expression of primed activated T cells.* Three colors flow cytometric intracellular staining analyses were performed with single-cell suspensions of lymphocytes of Peyer's Patches obtained from immunized mice 3 d after one immunization. Cells were stained with APC labeled with either anti-$CD8^+$ or anti-$CD4^+$ Abs, FICS labeled anti-$CD69^+$ Abs and PE labeled anti-IL-2 Abs and then analyzed and gated on live $CD8^+$ or $CD4^+$ T cells. B.) *Induction of CD8+ T cell specific cytotoxic activity.* Splenocytes were isolated from C57BL/6 mice after vaccination with either experimental or control DNA vaccines, 2 wk after challenge with D121 tumor cells, and analyzed for their cytotoxic activity in a ^{51} Cr-release assay at different effector-to target cell ratios. Specific lysis is shown mediated by $CD8^+$ T cell against

D121 tumor target cells Depicted are: cytotoxicity without depletion (×),specific lysis following depletoin of CD8+ T cell (■) or of CD4+ T cells (▲).Each value represents the mean of 8 animals. **, *P <0.001* compared to no T cell depletion. C.) *Production of IFN-γ.* This is indicated at the single-T cell level either without (□) or with stimulation (■) as determined by the ELISPOT assay and depicted by the number of immunspots formed per well. The mean spot distribution of each well in each experimental and control group is shown (n=4, mean±SD). **, *P <0.001, P<0.01, P<0.01* compared to empty vector, pIL-18 or pFra-1, respectively.

Fig. 4. T cell activation in nonlymphoid tissue. A.) *Up-regulated CTL markers.* Lymphocytes wereisolated from spleen, lungs, liver and blood of immunized mice 2wk (I) or 8 wk (II) after D2F2 (0.5×10^6) i.v. tumor cell challenge as well as 1 wk after re-challenge (III) of mice surviving after 14 wk. Mice treated only with empty vector served as controls. Two-color flow cytometric analyses were performed with single-cell suspensions of lymphocytes. PE-labeled anti-CD8 Ab were used in combination with FITC-conjugated anti-mouse CD3 mAb with each value representing the mean of 4 mice. Differences between the results obtained with the control group (empty vector) were statistically significant when compared to those of the treatment group (pUb-Fra-1/pIL-18). *P<0.05*, and especially significant in the group of animals where lymphocytes were obtained from lungs *P<0.001.* B.) *Interferon-γ release from lymphocytes in different tissues.* Lymphocytes were isolated as described above. Production of IFN-γ was detected at the single T-cell level by the ELISPOT assay. Each value represents lymphocytes from four mice. Differences between the control group (empty vector) and the treatment group (pUb-Fra-1/pIL-18) were statistically significant. *$P<0.05$, **$P<0.001$

Fig. 5. Maintenance of $CD8^+$ T memory cells in the absence of tumor antigen A.) The vaccination schedule of donor mice was the same as that shown in Fig. 2, i.e, 3 immunizations at 2 wk intervals, followed by i.v. challenge with 0.5×10^6 D2F2 tumor cells 1 wk after the last immunization. In the experimental group, 2d after re-immunization at wk 14 with pUb-Fra-1/pIL-18 or without re-immunization, lymphocytes were harvested from those mice that remained tumor free 2 months after the first tumor cell challenge. Lymphocytes (4×10^7 /mouse) were adoptively transferred from BALB/c to SCID mice, and 2d after transfer, these mice were challenged i.v. with 0.5×10^6 D2F2 tumor cells. Controls were mice adoptively transferred with lymphocytes harvested from control mice and immunized with only the empty vector or injected i.v. with PBS. Survival curves are representative of three separate experiments. B.) Fate of $CD8^+$ effector T cells after adoptive transfer into SCID mice was determined by 2-color flow cytometric analysis of $CD8^+$ (PE labeled); $CD3^+$(FITC labeled) splenic T cells as well as by the effect of apoptosis on these cells after parking them in these mice 7 and 30d, respectively. Differences between the two control groups (PBS and empty vector) and vaccine treatment groups were statistically significant. * *P<0.05,* ***P<0.001.*

Fig. 6. Turnover of memory $CD8^+$ T cells after vaccination with pUb-Fra-1/pIL-18 A.) Three colors flow cytometric analyses were performed of splenocytes obtained from immunized mice. Cells were stained with Cy-chrome labeled anti-$CD8^+$ Ab, PE-labeled anti-IL-2Rβ Ab and FITC labeled anti-$CD44^+$ Ab and then analyzed and gated on live $CD8^+$ T cell. *b.)* Lymphocytes were isolated from groups of BALB/c mice treated with either pIL-18 (□), pUb-Fra-1 (▩) and pUb-Fra-1/pIL-18 (▨) or from groups of control mice treated only with PBS(■) or empty vector (▩) obtained at 24h,72 h, 1 wk and 2 wk after tumor cell challenge and then analyzed by 3 color

flow cytometry, as described above. Each value represents the mean for eight mice. Differences between the two control groups (PBS and empty vector) and the vaccine treatment groups were statistically significant *$P<0.05$, **$P<0.001$. *c.)* Lymphocytes were isolated from spleen, lung, liver and blood of BALB/c mice of the treatment group pUb-Fra-1/IL-18(□) 8wk after tumor cell challenge or from mice in the control group, that were only immunized with the empty vector(▨) (I), or lymphocytes isolated from different tissues of SCID mice, adoptively transferred with either lymphocytes from mice immunized with pUb-Fra-1/pIL-18 (□)or with the empty vector(▨) 1 wk after tumor cell challenge (II). Three color flow cytometric analyses were performed and cells stained with Cy-chrome labeled anti-$CD8^+$ Ab, PE-labeled anti-IL-2Rβ Ab, and FITC-labeled anti-CD44 Ab. Data shown are gated on live $CD8^+$ T cells as described above. Each value represents the mean for 4 mice. Differences between the control and treatment groups of mice were statistically significant when compared to lymphocytes from lungs, blood and spleen of SCID mice after adoptive transfer following tumor cell challenge. This was also the case for lymphocytes from lungs and liver of syngeneic BALB/c mice obtained 8wk after tumor cell challenge. **$P<0.05$, *$P<0.001$.

Fig. 7. A.) IFN-γ release from $CD8^+$ effector T cells, adoptively transferred to SCID mice as measured on days 2, 7 and 30 after i.v. challenge with D2F2 tumor cells. IFN-γ production of each experimental and control group of mice by ELISPOT assay is shown (n=8, mean±SD). Differences between the control group (empty vector) and the treatment group (pUb-Fra-1/pIL-18) are statistically significant **$P<0.001$. B.) Cytotoxicity induced by $CD8^+$T cells, adoptively transferred to SCID mice. Splenocytes were isolated from mice, adoptively transferred with lymphocytes from successfully immunized BALB/c mice, and cytotoxity was measured on days

2, 7 and 30 after i.v. challenge with D2F2 tumor cells. Cytotoxicity was measured in a ^{51}Cr-release assay at different effector to target cell ratios. Each value shown represents the mean of 4 mice. Differences between the two control groups of empty vector (□) and PBS (○) and the vaccine treatment group (△) were statistically significant *$P<0.05$, **$P<0.001$

Acknowledgements

We thank C. Dolman and D. Markowitz for technical assistance; and K. Cairns for editorial assistance. H.Z. is a fellow of the Susan G.Komen Foundation. R.X. is supported by grants DAMD17-02-1-0137 and DAMD17-02-0562 from the Department of Defense. R.A.R. is supported by DOD grant BC031079; the Tobacco-Related Disease Research Program grant 12RT0002; and a grant from EMD-Lexigen Research Center, Billerica, MA. This is The Scripps Research Institute's manuscript number 16316-IMM.

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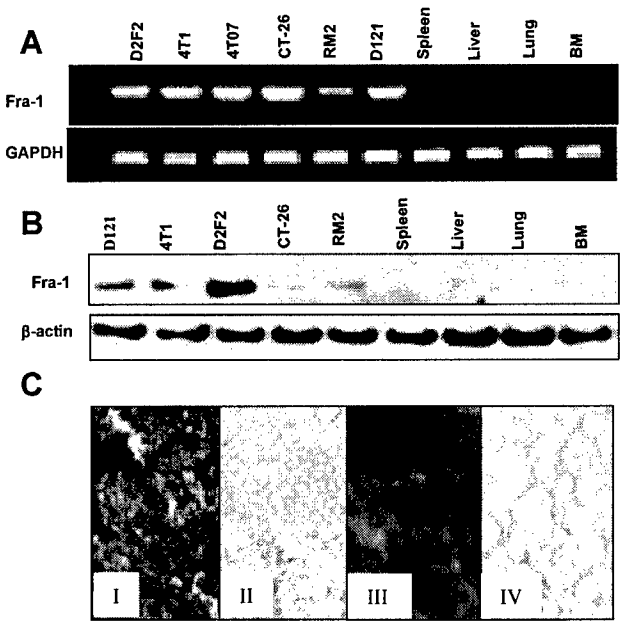
Fig1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4T1
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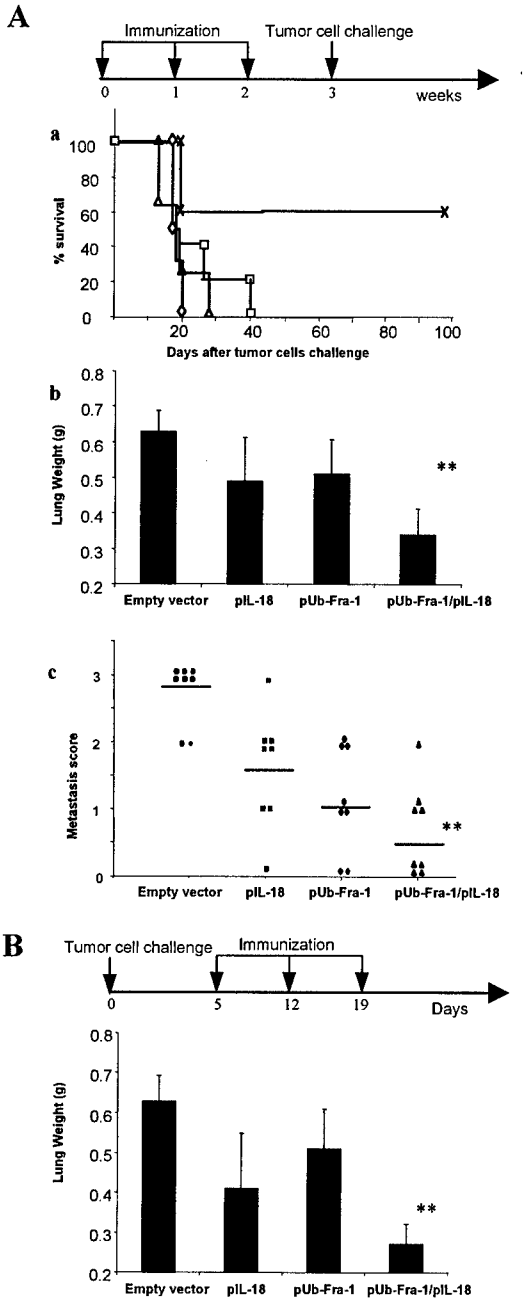
C
I
II
III
IV

Fig2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Tumor cell challenge
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1
2
3
weeks
a
% survival
Days after tumor cells challenge
b
Lung Weight (g)
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pUb-Fra-1
pUb-Fra-1/pIL-18
c
Metastasis score
**
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pUb-Fra-1
pUb-Fra-1/pIL-18
B
Tumor cell challenge
Immunization
0
5
12
19
Days
Lung Weight (g)
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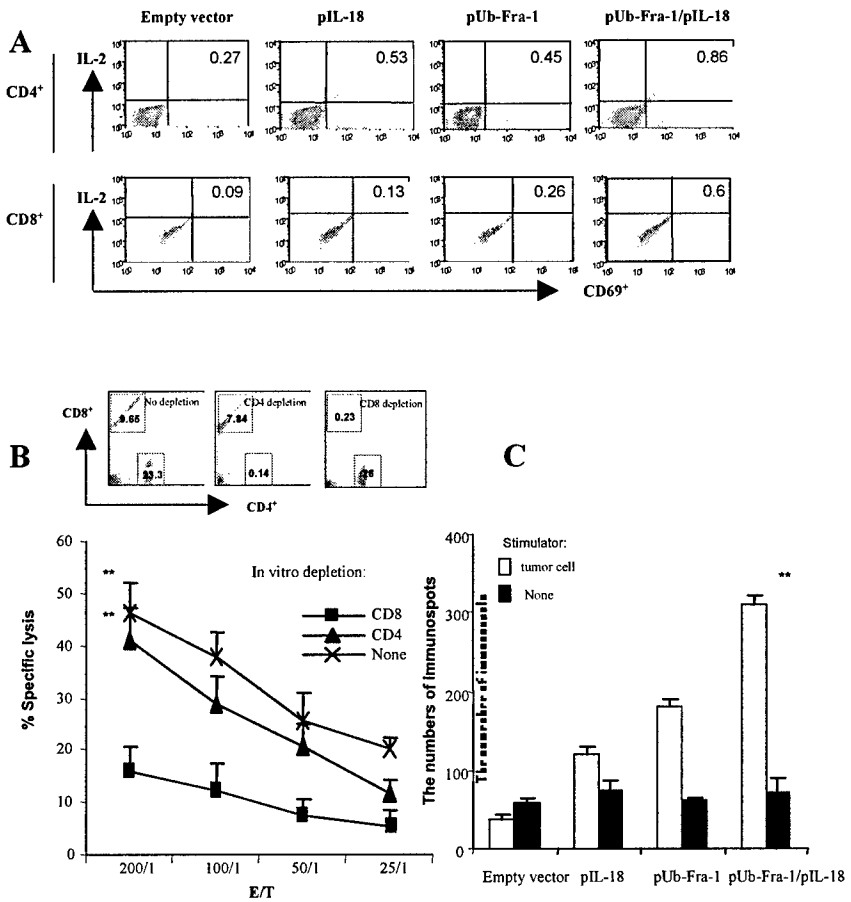
pUb-Fra-1/pIL-18

Fig3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0.86
CD8+
0.09
0.13
0.26
0.6
CD69+
B
CD8+
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CD4 depletion
CD8 depletion
0.65
7.84
0.23
0.14
CD4+
C
In vitro depletion:
CD8
CD4
None
% Specific lysis
E/T
200/1
100/1
50/1
25/1
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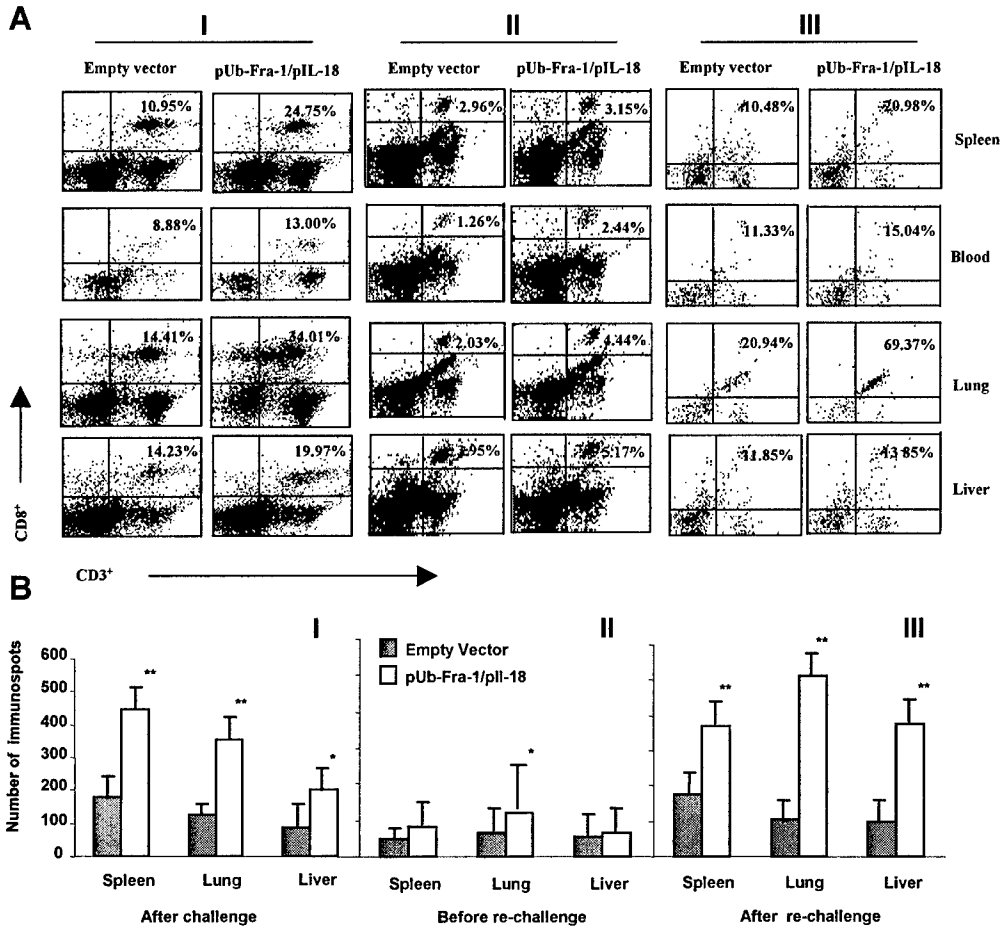
Fig4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24.75%
2.96%
3.15%
10.48%
20.98%
Spleen
8.88%
13.00%
1.26%
2.44%
11.33%
15.04%
Blood
14.41%
34.01%
2.03%
4.44%
20.94%
69.37%
Lung
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19.97%
2.95%
5.17%
11.85%
43.85%
Liver
CD8+
CD3+
B
Number of immunospots
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500
400
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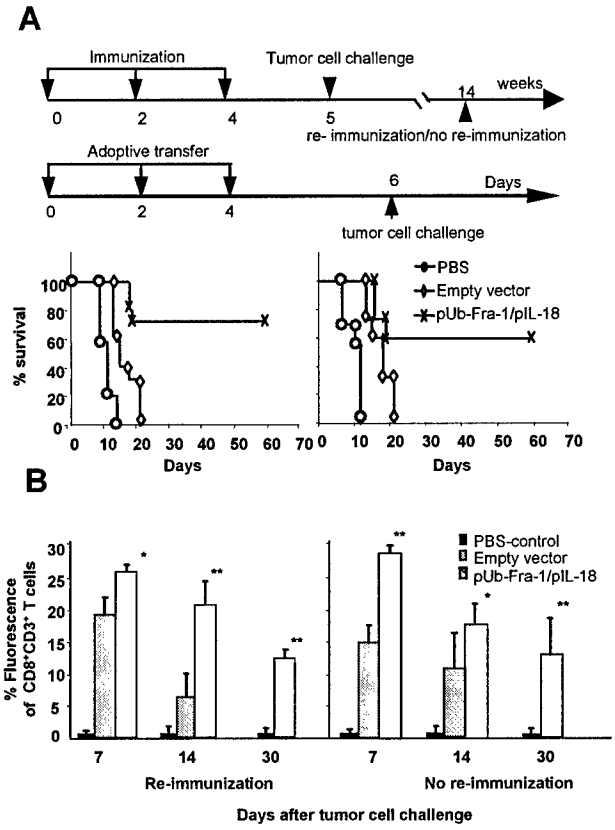
Spleen
Lung
Liver
After challenge
Before re-challenge
After re-challenge

Fig5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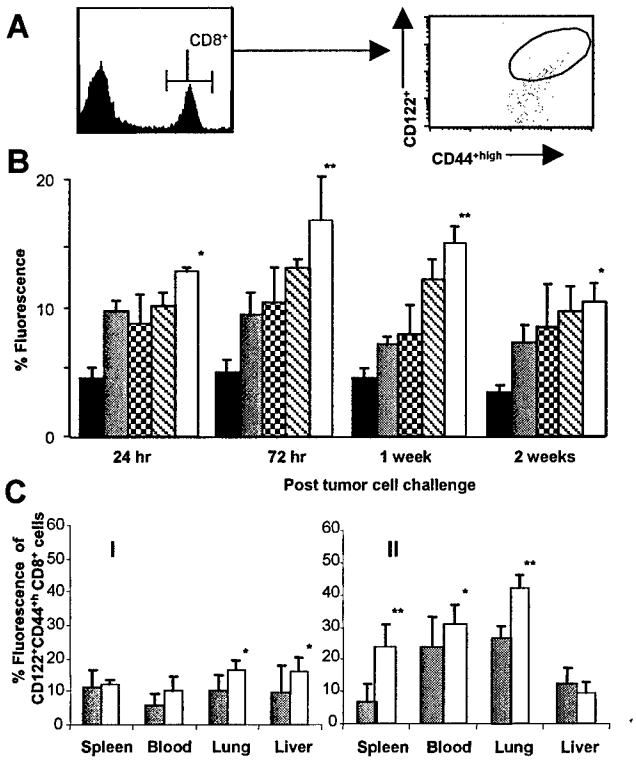
Immunization
Tumor cell challenge
weeks
0
2
4
5
14
re- immunization/no re-immunization
Adoptive transfer
6
Days
0
2
4
tumor cell challenge
PBS
Empty vector
pUb-Fra-1/pIL-18
% survival
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Days
0 10 20 30 40 50 60 70
Days
B
PBS-control
Empty vector
pUb-Fra-1/pIL-18
% Fluorescence of CD8+CD3+ T cells
30
25
20
15
10
5
0
*
**
**
**
*
**
7
14
30
7
14
30
Re-immunization
No re-immunization
Days after tumor cell challenge

Fig6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CD8+
CD122+
CD44+high
B
% Fluorescence
20
10
0
24 hr
72 hr
1 week
2 weeks
Post tumor cell challenge
C
% Fluorescence of CD122+CD44+high CD8+ cells
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II
60
50
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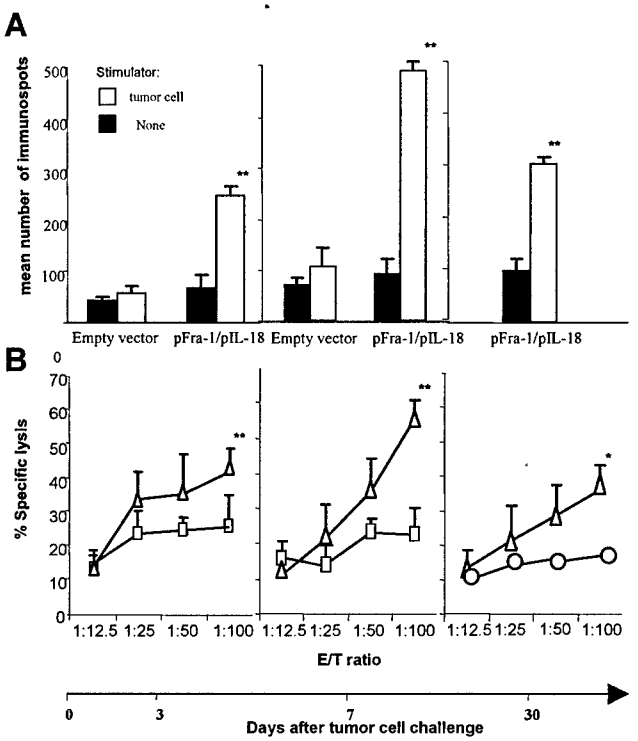
30
20
10
0
Spleen
Blood
Lung
Liver

Fig7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**
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Empty vector
pFra-1/pIL-18
pFra-1/pIL-18
B
% Specific lysis
0
70
60
50
40
30
20
10
0
**
**
*
1:12.5 1:25 1:50 1:100
1:12.5 1:25 1:50 1:100
1:12.5 1:25 1:50 1:100
E/T ratio
0
3
7
30
Days after tumor cell challenge